It Takes Two to Tango: DNA Polymerase Responses to Replication Stress in Human Cells

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Normal DNA

Microsatellites

National Institute on Aging
Laboratory of Molecular Gerontology
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The Human Genome

67% of the genome is repetitive sequence

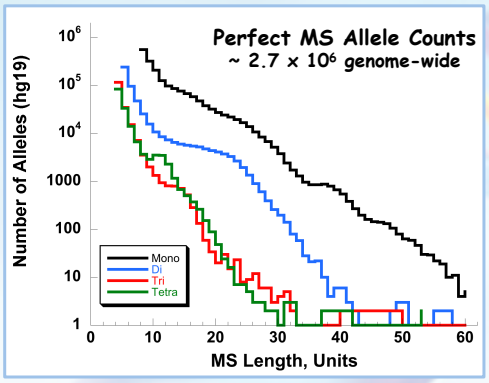
Repetitive sequences impact mutagenesis and genome evolution

Normal DNA

Microsatellites

The Human Genome

67% of the genome is repetitive sequence



Microsatellite Sequences

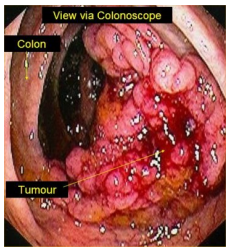
- Interspersed tandem repeats
- Repeat motif = 1-6 basepairs
- Genic and intergenic regions

How are repetitive sequences accurately and efficiently replicated in human cells?

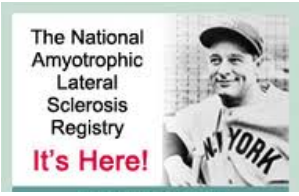
Microsatellites are Functional

Dog breed skeletal morphology (Runx-2 gene polymorphisms)

FMR-1 Repeat expansions



Colorectal cancer (MSI)



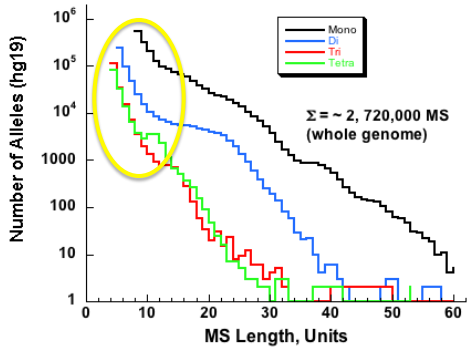
ATXN2 (SCA2) Interruptions

Genic Microsatellite Length Polymorphisms Affect Disease Risk

Gene	Allele	Effect	Disease	Reference
EGFR	$[CA]_{14-22}$ Intron 1	Transcription efficiency	Breast cancer	Gebhardt 1999 Buerger 2000 Buerger 2004
eNOS	$[CA]_{17-44}$ Intron 13	Splicing efficiency	Coronary artery disease	Stang 2000 Hui 2003; 2005
HO-1	$[CA]_{20-30}$	Promoter efficiency	Coronary artery disease	Chen 2002
CFTR	$[GT]_{9-13}$; $[T]_n$ Exon 9;	Splicing efficiency	Cystic fibrosis severity	Hefferson 2004
UGT1A1	$[TA]_{5-8}$; promoter	Transcriptional activity	Head/heck cancer; Gilbert's syndrome	Bosma 1995 Lacko 2010
IL28A	$[TA]_{10-18}$; promoter	Transcriptional activity	Response to IFN therapy	Sugiyama et al 2011

Lengths of microsatellite repeats directly affect mRNA production

How are microsatellite sequences accurately replicated in human cells?



~ 1.6 million genic microsatellites
(5' and 3' UTRs, promoters, introns, exons)

M.S. Hershey Medical Center

Millenium Science Building

PENNSTATE 1855

Eckert Laboratory
Penn State-Hershey
College of Medicine

Makova Laboratory
Penn State-University Park
College of Science

Experimental
- Biochemical
- Genetic

Computational
- Genomics
- Statistical models

What is a Microsatellite ?

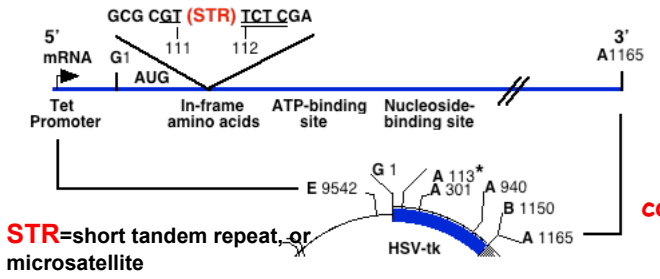
At what length does a tandem repeat become a microsatellite?

Definition based on mutational behavior

-combined computational and experimental approaches

Kelkar et al *Genome Biology and Evolution*. 2010; 2: 620-635.
Ananda, Walsh et al *Genome Biology and Evolution*, 2013; **5**: 606-620.

Microsatellite Mutagenesis – HSV-tk Forward Assay



STR=short tandem repeat, or microsatellite

STR alleles reflect common sequences and lengths present in human genome

Mutations detectable:

- Base substitutions
- Frameshifts (Indels)
- Deletions up to 5kB
- Rearrangements
- Microsatellite instability

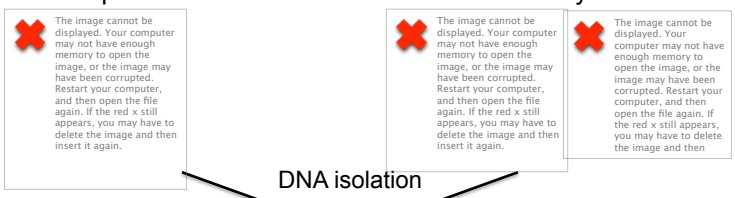
Mono	Di	Tetra
A/T	GT/CA	TTCC/AAGG
G/C	TC/AG	TTTC/AAAG
	AT/TA	TCTA/AGAT

Hile et al (2000) Cancer Research 60: 1698.

Overview of the HSV-tk Mutagenesis System

Genetic
Ex vivo (human cells)
DNA Replication

Biochemical
In vitro (Human proteins)
DNA synthesis



DNA isolation

HSV-tk Mutant Frequency (Quantitative)

↓

Mutational Specificity (Qualitative)

Definition of a Microsatellite

Mature Microsatellite

GTGTGTGTGT ⟷ GTGTGTGTGTGT

CACACACACA

GTGTGTGTGTGTGTGT

$[GT/CA]_n$

GTGTGTGTGTGTGTGTGTGT

Polymorphic allele

mutability

Threshold lengths
Mono: 9 units
Di: 5 units
Tri: 4 units
Tetra: 4 units

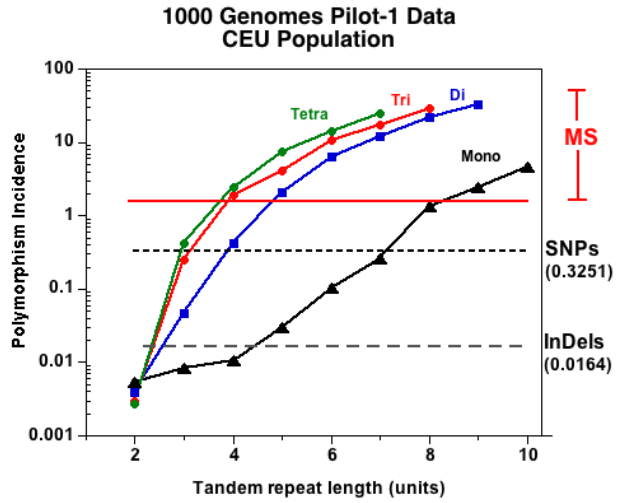
↑ "birth"

GTGTGTTTGT
CACACAAACA

Short tandem repeat

Kelkar et al *Genome Biology and Evolution*. 2010; 2: 620-635.
Ananda, Walsh et al *Genome Biology and Evolution*, 2013; **5**: 606-620.

Microsatellites are Highly Polymorphic in Human Populations



- Polymorphism incidence used as a proxy for microsatellite mutability

G. Ananda, E. Walsh et al., Genome Biol. Evol. (2013) 5(3):606-20

Microsatellites are Stable in Mitotic Human Cells

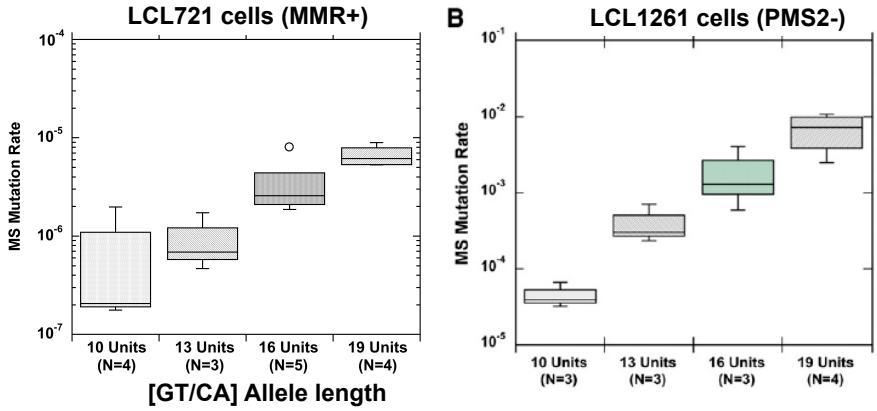
Variable

	Motif Sequence	Mutation Frequency per cell generation x 10^{-6}	
		Median	Range
motif size	$[G/C]_{10}$	29	14-34
	$[GT/CA]_{10}$	**0.21**	0.18-2.0
	$[GT/CA]_{16}$	3.4	2.1-11
Number (length)	$[TC/AG]_{11}$	2.3	0.59-7.5
	$[TC/AG]_{17}$	9.8	6.0-28
	$[TC/AG]_{20}$	21	16-25
sequence	$[TTCC/AAGG]_9$	5.6	3.8-20
	$[TTTC/AAAG]_9$	35	12-57
	$[TCTA/AGAT]_9$	**48**	36-110

Measurements performed using LCL721 cell line, derived from a normal donor

Eckert et al (2002); Eckert and Hile (2009)

Mature Microsatellite Mutation Rates



- **Mismatch repair stabilizes microsatellites, but also introduces mutational biases**

Baptiste et al (2013) G3: Genes, Genomes, Genetics; Shah and Eckert (2009) Cancer Research

It takes a village to raise a child….

Multiple DNA polymerases are required to maintain human genome stability

Replication

Pol alpha (α, *POLA*)
Pol delta (δ, *POLD1*)
Pol epsilon (ε, *POLE*)

Repair

Pol beta (β, *POLB*)
Pol lambda (λ, *POLL*)
Pol mu (μ, *POLM*)
Pol theta (θ, *POLQ*)

Specialized Synthesis

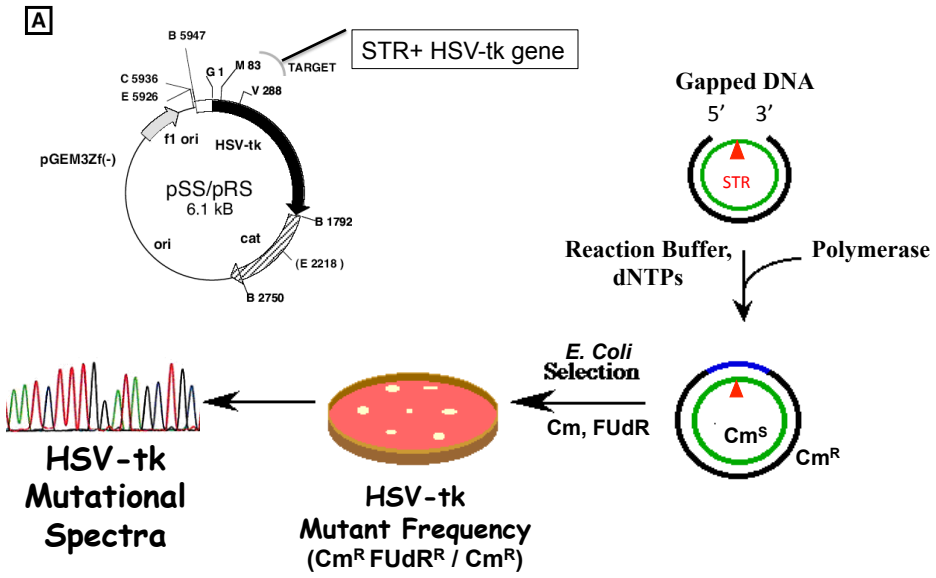
Pol zeta (ζ, *POLZ; REV3*)
Pol eta (η, *POLH; XPV*)
Pol kappa (κ, *POLK; DINB1*)
Pol iota (ι, *POLI*)
Rev 1 (dCMP transferase)
Pol nu (ν, *POL N*)

Mitochondrial

Pol gamma (γ, *POLG*)

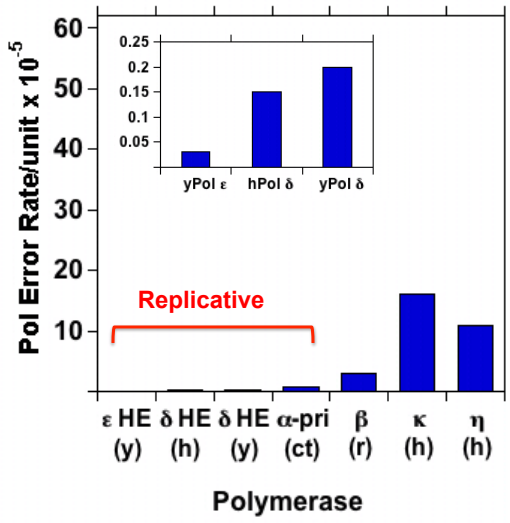
Are specialized polymerases required for accurate replication of repetitive DNA ?

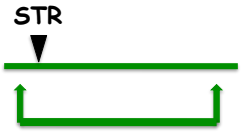
In vitro HSV-tk Polymerase Assay



Eckert, Mowery and Hile (2002) Biochemistry 41: 10490-98.

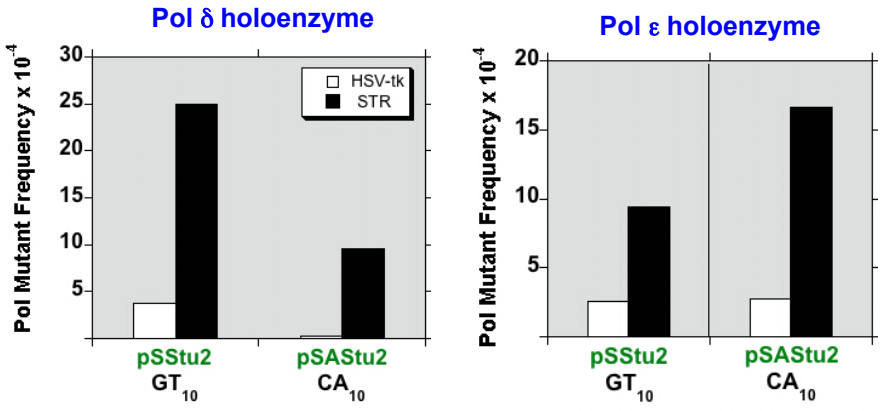
Replicative Polymerases Display High Accuracy within the HSV-tk Gene





HSV-tk Sequences (frameshift monitors)

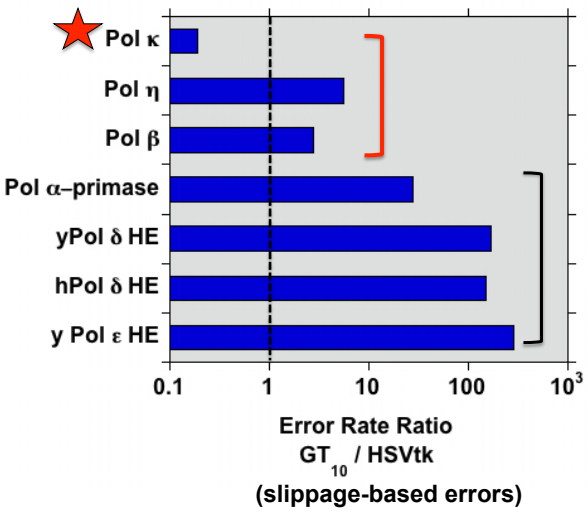
Replicative holoenzymes are highly inaccurate within microsatellites

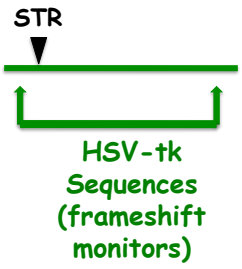


- The 3' to 5' proofreading exonuclease does <u>not</u> remove microsatellite indel errors

Abdulovic, Hile, Kunkel and Eckert (2011) DNA Repair 10: 497.

Specialized polymerases are more accurate for motif-based InDel errors





Hile et al. (2011) Nucl. Acids Res. 40: 1636

Microsatellite Mutational Specificity

$[GT/CA]_9 \longleftarrow [GT/CA]_{10} \longrightarrow [GT/CA]_{11}$

Base Deletion Error

$[GT]_4\ T\ [GT]_5$

Base Substitution Error

$[GT]_4\ TT\ [GT]_5$

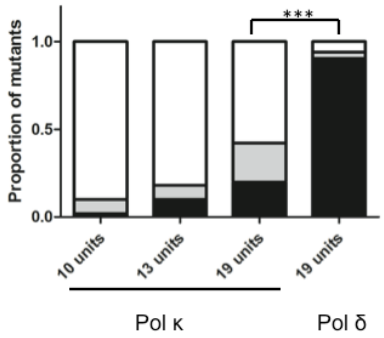
Base Insertion Error

$[GT]_5\ G\ [GT]_5$

Microsatellite Interruptions

Pol κ Displays a Unique Error Specificity within Microsatellites

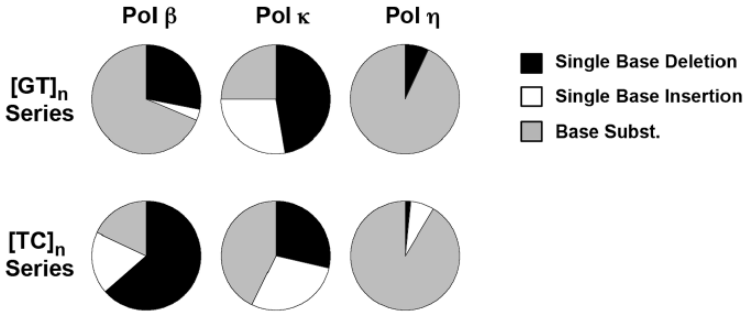
White bars: HSVtk gene errors
Black bars: Microsatellite errors
*** $p=2.9 \times 10^{-4}$ (Fisher's)



Observed for di- and tetranucleotide microsatellites

Hile et al *Nucleic Acids Res.* 2012; 40: 1636-47
Baptiste and Eckert *Environ. Mol. Mutagenesis* 2012; **53:** 787-796.

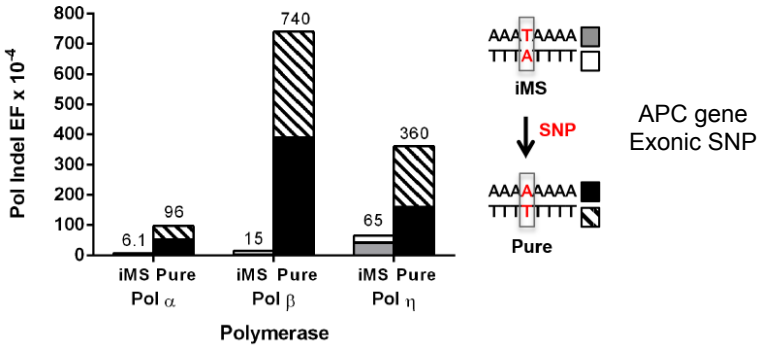
Specialized DNA Polymerases Create Interruption Errors



The specificity of in vitro polymerase errors is in concordance with genome-wide analyses of interrupted microsatellites within individual human genomes

Ananda et al. *PLoS Genetics*, 2014; 10(7):e1004498.

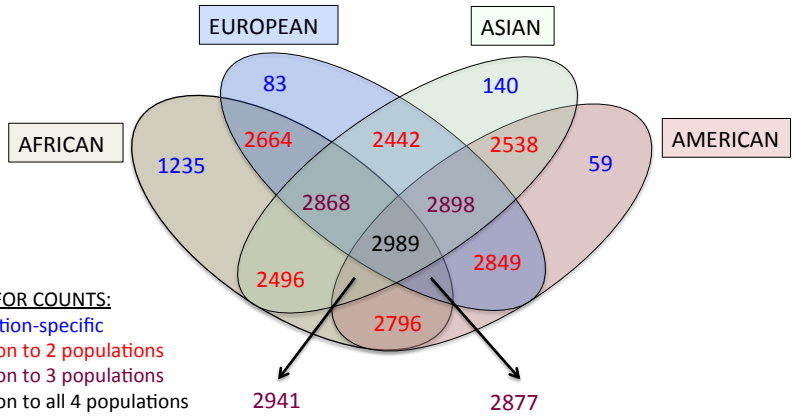
Interruptions Decrease Polymerase Errors



Interrupting base is rarely deleted

Ananda et al. *PLoS Genetics*, 2014; 10(7):e1004498.

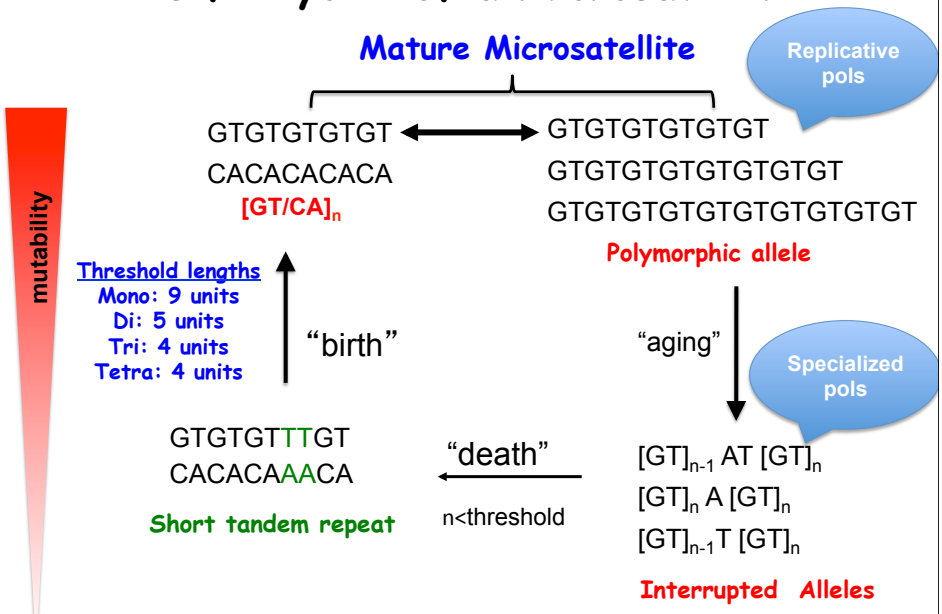
Microsatellite interruptions occur within individual genomes



COLORS FOR COUNTS:
1. Population-specific
2. Common to 2 populations
3. Common to 3 populations
4. Common to all 4 populations

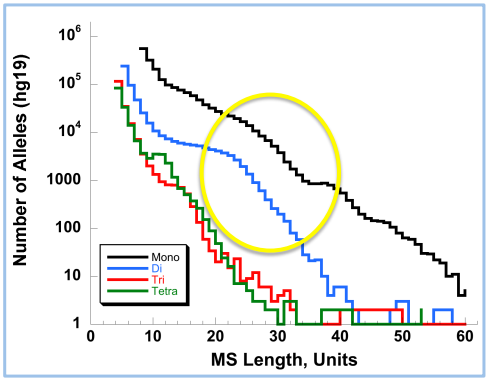
~11% of interrupted microsatellites lie within exons (UTRs + coding)

"Life Cycle" of a Microsatellite



Ananda et al. *PLoS Genetics*, 2014; 10(7):e1004498.

How are microsatellite sequences efficiently replicated in human cells?



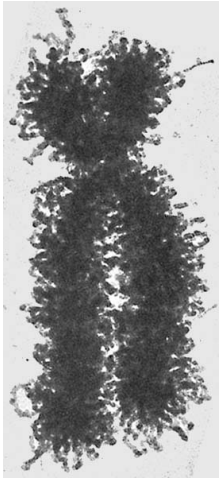
Chromosome Fragile Sites

Telomere

Rare fragile sites

Common fragile sites (CFS)*

Interstitial Telomere



$[TTAGGG]_n$

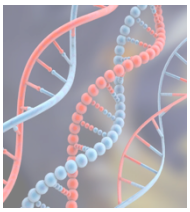
$[CGG]_n$

$[A/T]_n$
$[AT/TA]_n$

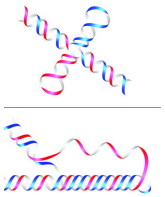
Microsatellite DNA

* Low complexity A/T-rich sequences and mononucleotide A/T microsatellites are significant predictors of aphidicolin-induced CFS genome-wide.
Fungtammasan et al. (2012) Genome Research 22: 993-1005.

Repetitive Sequences Favor Non-B Form DNA Secondary Structures



B-DNA ⟷ **Non B-DNA**



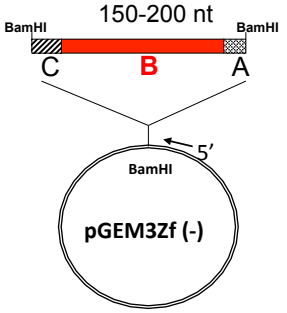
B-DNA structure is favored in random sequences

Non-B DNA Structures

- Slipped strands
- Bent DNA $(A)_n$ $(T)_n$
- Triplex (H) $(TC)_n$ $(AAG)_n$
- Cruciforms $(AT)_n$
- Z-DNA $(GT)_n$ $(GC)_n$
- Hairpins $(AT)_n$ $(CAG)_n$
- G4 $(TTAGGG)_n$

In Vitro Assay for DNA Polymerase Pausing

Template	CFS	Repeat Elements
AT repeat 1	FRA16D	$[A/T]_{28}$, $[AT/TA]_{23i}$
AT repeat 2	FRA16D	$[A/T]_{9}$, $[AT/TA]_{8}$
AT repeat 3	FRA3B	$[A/T]_{22}$, $[AT/TA]_{25}$
AT repeat 4	FRA16D	$[AT/TA]_{9}$
Quasipalindrome 1	FRA16D	$[A/T]_{19}$, QP_{36}
Quasipalindrome 2	FRA16D	QP_{37}
Quasipalindrome 3	FRA16D	QP_{29}

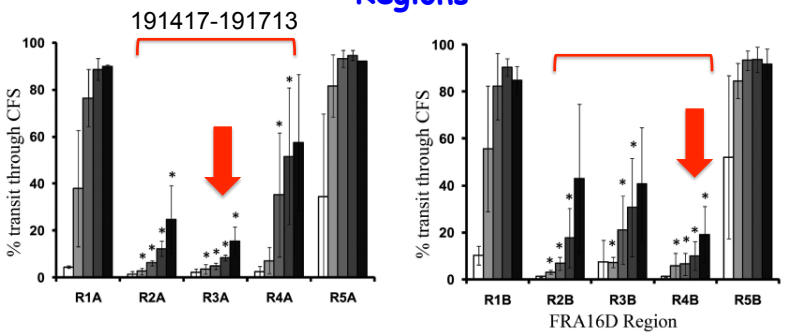


A = constant proximal
B = CFS sequence
C = constant distal

% Transit = ∑ Products C/B+C

Shah et al. (2010) Nucl. Acids Res. 38: 1149-1162.
Walsh et al. (2013) J. Mol. Biol. 425: 232-43.

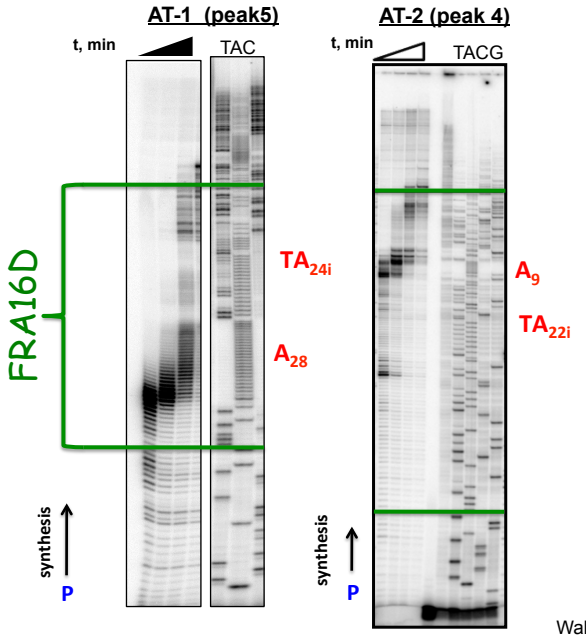
Slowed Elongation by Human Polδ4/PCNA within FRA16D Regions

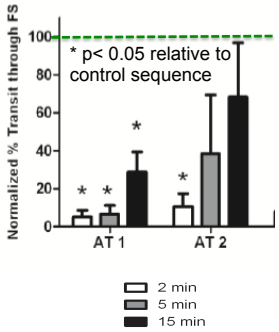


- Slowed DNA synthesis within specific sequences
- Strand biases observed
- Similar results observed in HeLa cell extracts

Shah et al (2010) Nucl. Acids Res.38: 1149.

Pol δ4 Inhibition: A/T and AT/TA Microsatellites

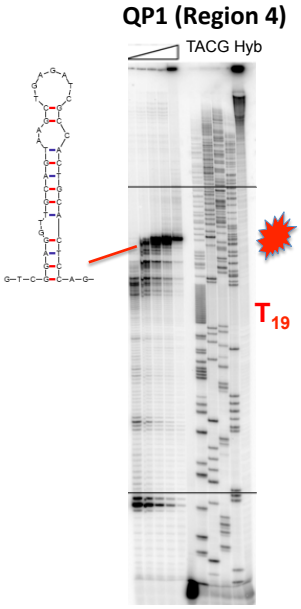




Control= sequence from FRA16D with high %AT but no repeated motifs

Walsh et al. (2013) J. Mol. Biol. 425: 232.

Pol δ4 Inhibition: Quasipalindromes

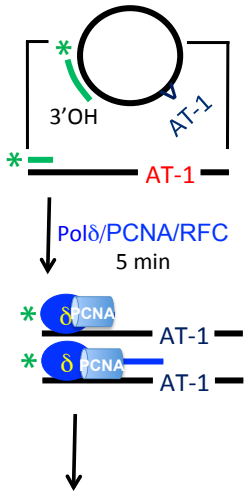


Motif	Stem length (bp)	Stem self-homology	Loop length (nt)	Pol δ TP
QP1	29	76 %	7	0.51
QP2	34	82%	3	0.38
QP3	20	100 %	9	0.72

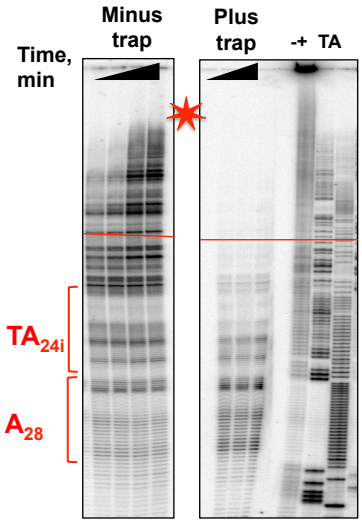
TP = termination probability at base of QP

Walsh et al. (2013) J. Mol. Biol. 425: 232.

hPol δ4/PCNA Holoenzyme Dissociates at Inhibitory Sequences



Add cold ssDNA to trap dissociated pol
Monitor synthesis



Walsh et al. (2013) J. Mol. Biol. 425: 232.

CFS Motifs Inhibitory to Replicative Polymerases

Sequence Motif*	Replicative Polymerase		
	Pol δ4	ε (cat)	α–primase
Microsatellites			
$[A/T]_9$	+/-		
$[A/T]_{19}$	+	+	+
$[A/T]_{22}$	+		
$[A/T]_{28}$	+++	+/-	++
$[AT/TA]_8$	–		
$[AT/TA]_{24i}$	+/-	++	++
$[AT/TA]_{25}$	++		
Inverted repeats 29 nt stem (100%)	+++		
36 nt stem (75%)	+++	+++	++
37 nt stem (82%)	+++		

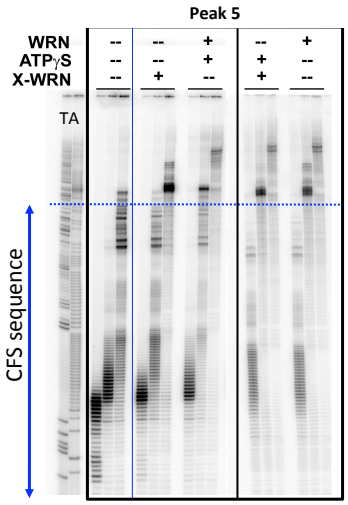
Consistent with genome-wide features

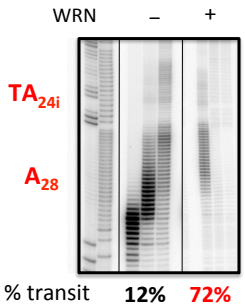
* Strand biases observed

Do specific *trans*-acting replication proteins alter DNA synthesis progression?

Polymerase-interacting proteins (RecQ helicases)

WRN facilitates hPol δ4 progression: Microsatellites

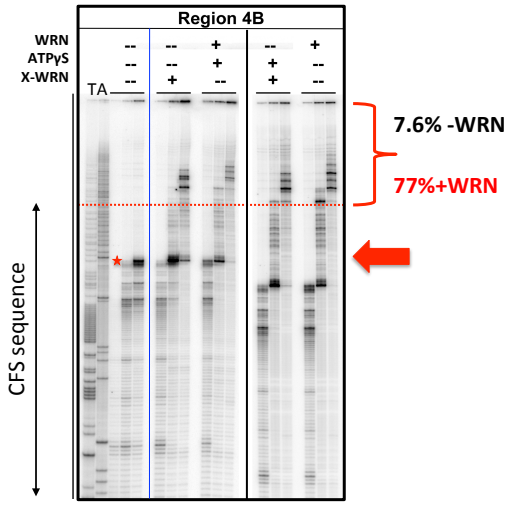




Neither helicase nor exonuclease activities of WRN are necessary for pol δ stimulation

Shah et al (2010) Nucl. Acids Res.38: 1149.

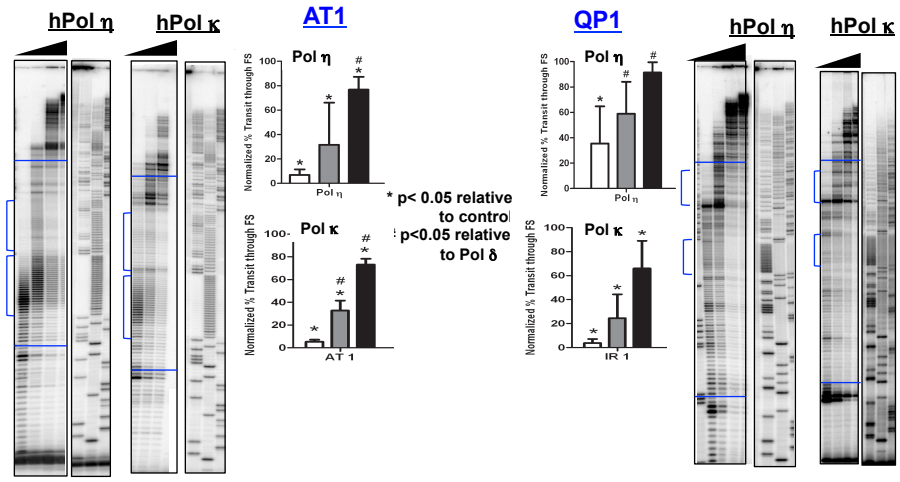
WRN facilitates hPol δ4 progression: Hairpin Structure



- WRN has no effect on pol δ synthesis efficiency through control sequences
- WRN effect due to direct interaction with either Pol δ and/or the DNA template

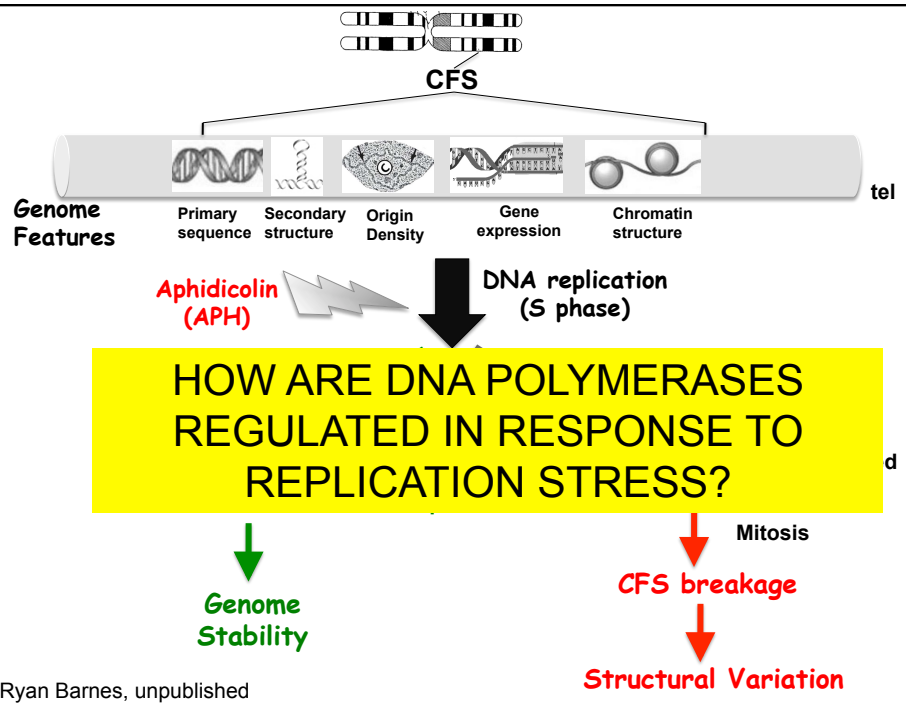
Do specific *trans*-acting replication proteins alter DNA synthesis progression?

Y Family polymerases display efficient synthesis through CFS repeat elements



Common 3 polymerase reaction conditions:
KPO_4 buffer (pH7.6)
80% extension of control template

Bergoglio et al. (2013) J. Cell Biol. 201: 395-408.
Walsh et al. (2013) J. Mol. Biol. 425: 232-243.



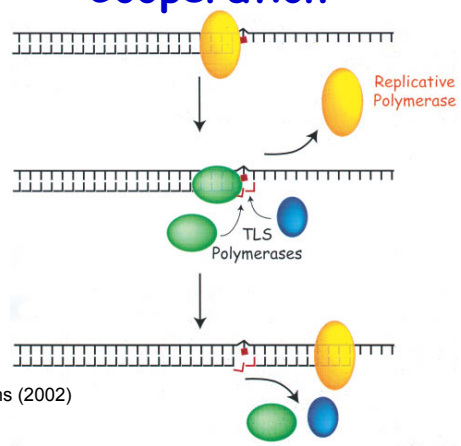
Ryan Barnes, unpublished

Diverse functions of DNA Polymerase η

1. During natural, targeted mutation
   - immune cell somatic hypermutation
2. At DNA lesions during replication (translesion synthesis)
   - UV photoproducts; cis-platinum adducts
3. **Common fragile site replication**

It Takes Two: DNA Polymerase Cooperation

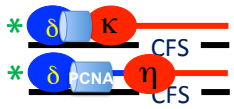
General model



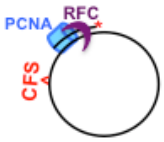
Taken from: Pages and Fuchs (2002)

Does polymerase exchange take place at inhibitory CFS-derived sequences?

In vitro assay to examine DNA polymerase cooperation



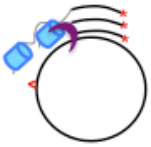
(1) RFC loading of PCNA onto primed ssDNA templates



Add Pol δ4

37°C, 5 min

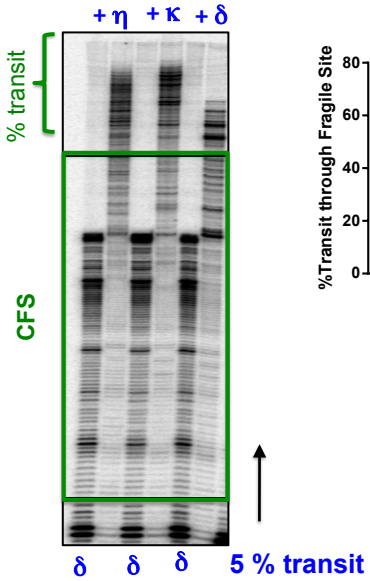
(2) Pol δ HE formation and initial synthesis

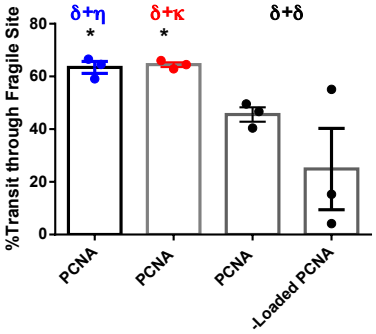


Equal activity
+ Pol η (κ)
-or-
+ Pol δ4 (control)

Monitor synthesis At 15 min (T_2)

Specialized Polymerases Efficiently Exchange with a Moving Pol δ Holoenzyme



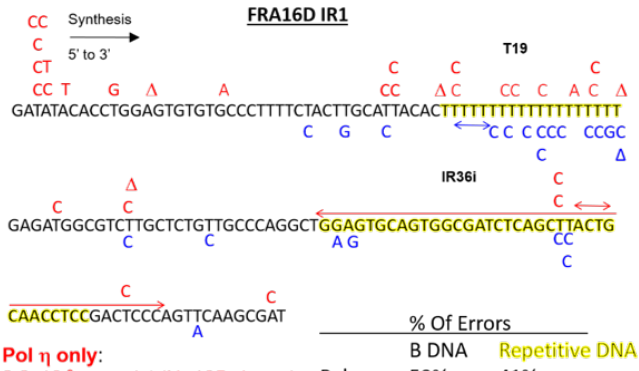


*Significantly different from δ control reactions; P<0.05

N= 3 independent reactions

Mono- Pol η reaction
% transit = 38%

Pol η Error Signatures are Present at CFS Inhibitory Motifs in Dual Polymerase Reactions

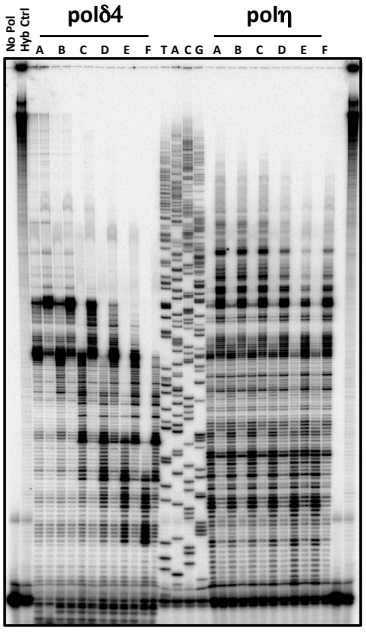


Pol η only:
2.2x10^{-3} errors/nt (N=105 clones)
Pol δ only:
0.1x10^{-3} errors/nt (N=60 clones)
Pols η+δ:
1.7 x 10^{-3} errors/nt (N=94 clones)

	% Of Errors	
	B DNA	Repetitive DNA
Pol η	59%	41%
Pol δ + η	25%	75%

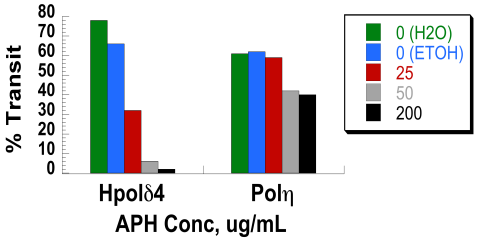
Fishers exact test – P=0.017

Hpolδ4, but not Polη is Inhibited by APH

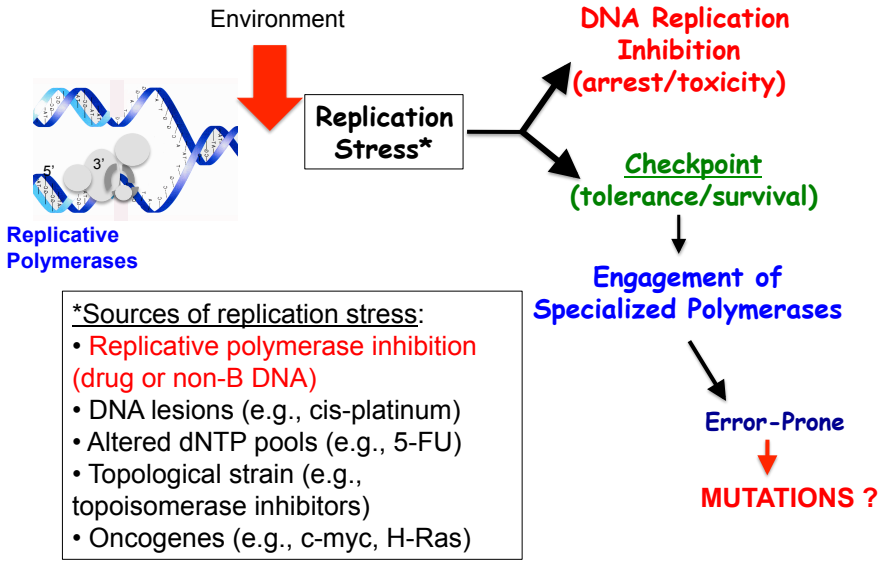


A = H_2O control
B = ETOH control
C = 25ug/mL APH
D = 50ug/mL APH
E = 100ug/mL APH
F = 200ug/mL APH

Template = pGEM 3Zf (-) +PCNA/RFC



Replication Stress and Genome Instability



*Sources of replication stress:
- Replicative polymerase inhibition (drug or non-B DNA)
- DNA lesions (e.g., cis-platinum)
- Altered dNTP pools (e.g., 5-FU)
- Topological strain (e.g., topoisomerase inhibitors)
- Oncogenes (e.g., c-myc, H-Ras)

It takes a village to raise a child….

Multiple DNA polymerases are required to maintain human genome stability

Replication

Pol alpha (α, *POLA*)*
Pol delta (δ, *POLD1*)*
Pol epsilon (ε, *POLE*)*

Repair

Pol beta (β, *POLB*)*
Pol lambda (λ, *POLL*)
Pol mu (μ, *POLM*)
Pol theta (θ, *POLQ*)

Specialized Synthesis

Pol zeta (ζ, *POLZ; REV3*)
Pol eta (η, *POLH; XPV*)*
Pol kappa (κ, *POLK; DINB1*)*
Pol iota (ι, *POLI*)*
Rev 1 (dCMP transferase)
Pol nu (ν, *POL N*)
PrimPol *

Mitochondrial

Pol gamma (γ, *POLG*)

* Studied in the Eckert laboratory

Perspective

The number and wide ranging activities of mammalian DNA polymerases may reflect the biochemical complexity of completing replication of a repeat-dense genome.

The Team

Eckert Laboratory

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- Erin Walsh
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- Patricia Opresko (WRN)
- Thomas Kunkel (yPolδ,ε)
- Linda Bloom (RFC)
- Marila Cordeiro-Stone; G. Lucian Moldovan (cell lines)

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